A comparative proteomic study of plasma in feline pancreatitis and pancreatic carcinoma using 2-dimensional gel electrophoresis to identify diagnostic biomarkers: A pilot study

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Abstract

While pancreatitis is now recognized as a common ailment in cats, the diagnosis remains challenging due to discordant results and suboptimal sensitivity of ultrasound and specific feline pancreatic lipase (Spec fPL) assay. Pancreatitis also shares similar clinical features with pancreatic carcinoma, a rare but aggressive disease with a grave prognosis. The objective of this pilot study was to compare the plasma proteomes of normal healthy cats (n = 6), cats with pancreatitis (n = 6), and cats with pancreatic carcinoma (n = 6) in order to identify potential new biomarkers of feline pancreatic disease. After plasma protein separation by 2-dimensional gel electrophoresis, protein spots were detected by Coomassie Brilliant Blue G-250 staining and identified by mass spectrometry. Alpha-1-acid glycoprotein (AGP), apolipoprotein-A1 (Apo-A1), and apolipoprotein-A1 precursor (Pre Apo-A1) appeared to be differentially expressed, which suggests the presence of a systemic acute-phase response and alteration of lipid metabolism in cats with pancreatic disease. Future studies involving greater case numbers are needed in order to assess the utility of these proteins as potential biomarkers. More sensitive proteomic techniques may also be helpful in detecting significant but low-abundance proteins.

Résumé

Bien que la pancréatite soit maintenant reconnue comme un problème peu fréquent chez les chats, le diagnostic demeure un défi étant donné les résultats discordants et la sensibilité sous-optimale de l'échographie et de l'épreuve spécifique de la lipase pancréatique féline (Spec fPL). La pancréatite partage également des similarités cliniques avec le carcinome pancréatique, une maladie rare mais agressive ayant un pronostic grave. L'objectif de cette étude pilote était de comparer les protéomes plasmatiques de chats normaux en santé (n = *6), de chats avec une pancréatite* (n = *6), et de chats avec un carcinome pancréatique* (n = *6) afin d'identifier de nouveaux biomarqueurs potentiels de maladie pancréatique féline. Après séparation des protéines plasmatiques par électrophorèse en gel en deux dimensions, les taches de protéines furent détectées par coloration avec du bleu brillant de Coomassie G-250 et identifiées par spectrométrie de masse. La glycoprotéine acide alpha-1 (AGP), l'apolipoprotéine A1 (Apo-A1), et le précurseur de l'apolipoprotéine A1 (Pre Apo-A1) apparaissent comme étant exprimées de manière différentielle, ce qui suggère la présence d'une réponse de phase-aiguë systémique et une altération du métabolisme des lipides chez les chats avec une maladie pancréatique. Des études additionnelles regroupant un plus grand nombre de cas sont nécessaires afin d'évaluer l'utilité de ces protéines comme biomarqueurs potentiels. Des techniques plus sensibles de protéomique pourraient également être utiles pour détecter des protéines significatives mais de faible abondance.*

(Traduit par Docteur Serge Messier)

Introduction

Disease biomarkers have been widely used in human medicine for both diagnostic and monitoring purposes and their usage in companion animals is steadily increasing (1–3). As such, discovery of new biomarkers is highly desirable. Proteomics is a valuable tool in this regard due to its ability to reveal differentially regulated or modified proteins that may act as potential biomarkers (3).

Pancreatitis is now recognized as a common entity in cats (4–6). The specific feline pancreatic lipase (Spec fPL) assay and pancreatic ultrasonography are widely regarded as the most useful antemortem diagnostic tests (6–8). Misdiagnosis is still a concern, however, as sensitivities of 79% and 67% and specificities of 82% and 33%, respectively are suboptimal and may lead to false negative and false positive results (8,9). Furthermore, agreement between the Spec fPL assay and pancreatic ultrasonography is only fair, with discordant results in 20% to 36% of cases (10,11). As pancreatic biopsy is rarely used for histological evaluation in general practice (5), the development of an additional noninvasive biomarker would be valuable in challenging cases with discordant or suspected erroneous results. An ideal biomarker would also be able to distinguish pancreatitis from other exocrine pancreatic diseases, such as pancreatic carcinoma. Although

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Table I. Diagnostic tests carried out on cats with pancreatic carcinoma and pancreatitis

Diagnosis	Age (y)	Gender	Diagnostic tests
Carcinoma	3	FS	Histopathology, Spec fPL
Carcinoma	7	FS	Histopathology
Carcinoma	11	MN	Cytology, ultrasound
Carcinoma	11	MN	Histopathology, ultrasound (negative)
Carcinoma	16	MN	Histopathology, Spec fPL
Carcinoma	18	FS	Cytology, ultrasound, Spec fPL
Pancreatitis	2	MN	Spec fPL
Pancreatitis	9	MN	Ultrasound, Spec fPL
Pancreatitis	14	FS	Spec fPL
Pancreatitis	14	FS	Histopathology, ultrasound
Pancreatitis	18	FS	Spec fPL
Pancreatitis	18	FS	Histopathology, ultrasound, Spec fPL

FS — female spayed; MN — male neutered; Spec fPL — specific feline pancreatic lipase.

relatively rare, feline pancreatic carcinoma often presents similarly to pancreatitis, with vague, nonspecific clinical signs (12–14). The Spec fPL value may also be elevated secondary to tumor-induced pancreatic inflammation (8,12). Despite the initial resemblance, pancreatic carcinoma is a very aggressive disease, with median survival of only 7 to 165 d (12,13). A biomarker able to differentiate pancreatitis from pancreatic carcinoma would allow veterinarians to better advise owners regarding patient care and expected outcomes.

Proteomic research using serum and plasma samples has identified several proteins that may act as potential biomarkers for diagnosing and differentiating between pancreatitis and pancreatic carcinoma in humans (15–17). To date, only a limited number of proteomic studies has been conducted in cats, concentrating on the urine proteome, degenerative joint disease, and hypertrophic cardiomyopathy (1,2,18,19). While 1 study has also evaluated the serum protein profile of normal healthy cats (20), feline pancreatic disease remains unexplored.

Plasma is an economical and noninvasive sample in which the proteomic patterns may be indicative of feline pancreatic health. The objective of this study was to compare the plasma proteomes of healthy control cats and cats with pancreatic disease (pancreatitis and pancreatic carcinoma) for potential biomarker identification.

Materials and methods

Cats

The study population consisted of 18 client-owned cats: 6 healthy controls, 6 with pancreatitis, and 6 with pancreatic carcinoma. A board-certified specialist in internal medicine physically examined the healthy controls and no significant abnormalities were found on hematology, biochemistry, and urinalysis profiles. In this group, 2 cats were neutered males, 4 were spayed females, and all were 8 to 17 y of age (median 9 y). Cats with pancreatic disease were examined by licensed veterinarians at 7 veterinary hospitals across western Canada. Cats diagnosed with pancreatitis had supportive clinical signs and 1 or more of the following: a Spec fPL value of ≥ 3.6 μg/L, positive ultrasound findings, or histopathologic confirmation. The diagnosis of cats with pancreatic carcinoma was confirmed by cytology or histology. Gender, neuter status, age, and specific diagnostic tests used for cats with pancreatic disease are reported in Table I.

Plasma collection

Blood was collected into sodium-citrated tubes (3.2% sodium citrate; BD Vacutainer, Franklin Lakes, New Jersey, USA) by referring veterinarians at multiple small animal clinics across western Canada. After centrifugation for 10 min, the plasma was separated and stored frozen at −20°C until shipment to the Western College of Veterinary Medicine (WCVM), University of Saskatchewan, Saskatoon. When received, all plasma samples were immediately stored at −80°C until use. The protein concentration of all samples was determined by the Bradford method (21).

2-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE)

Electrophoretic separation of plasma proteins was conducted as described in a previous study (22). Briefly, 400 μg of unfractionated plasma protein was mixed with rehydration buffer {8 mol/L urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% immobilized pH gradient buffer 4 to 7 (GE Healthcare, Uppsala, Sweden), and 2.8% 1,4-dithiothreitol} to a final volume of 250 μL. The mixture was applied to immobilized pH gradient gel strips (13 cm, pH 4 to 7, Immobiline DryStrip; GE Healthcare) and rehydrated at 50 V for 12 h (Ettan IPGphor II Isoelectric Focusing Unit; GE Healthcare). Isoelectric focusing conditions were 500 and 1000 V for 2 h each (1 h linear voltage ramping, then maintenance at that voltage for 1 h), followed by 8000 V (1 h linear voltage ramping, followed by maintenance at 8000 V for 12 kVh).

After isoelectric focusing to separate proteins based on their isoelectric point (pI), proteins were separated by their molecular weight. Gel strips were equilibrated in equilibration buffers [6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulphate, and 50 mmol/L Tris-hydrochloride (HCl) pH 8.8] with 1% 1,4-dithiothreitol for the first step and 2.5% iodoacetamide for the second step. Strips were then placed on the top of pre-cast 12.5% polyacrylamide gels. In order to estimate the molecular weight of separated proteins, 15 μL of a marker solution containing known standards (Broad-Range SDS-PAGE Standards; Bio-Rad Laboratories, Richmond, California, USA) was added to the left of the gel strip. Electrophoresis was carried out at 100 V for 1 h, then at 225 V until 30 min after the tracking

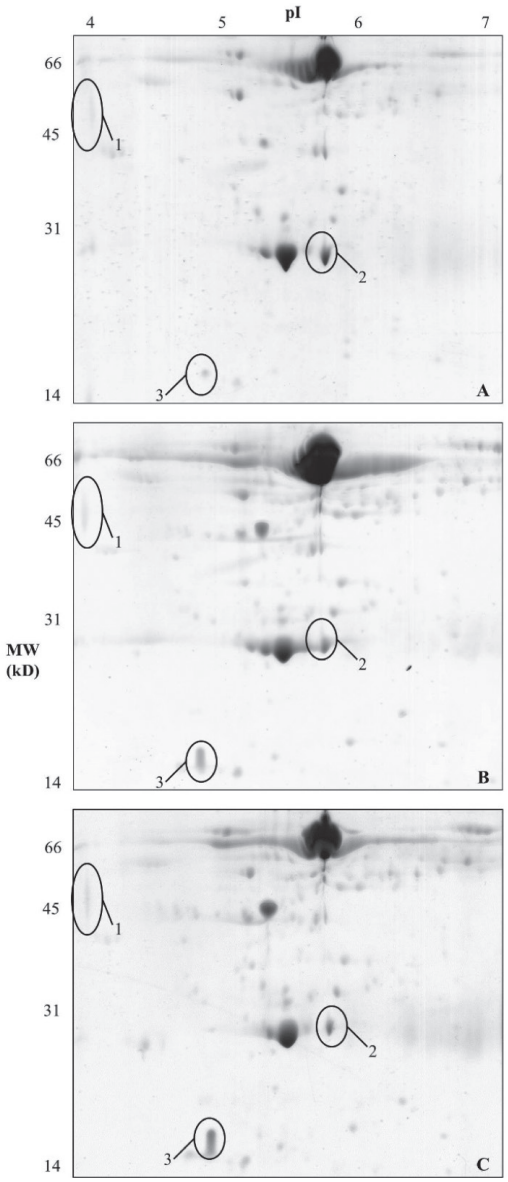

Figure 1. Representative images of Coomassie-stained plasma protein profiles of healthy control cats (A), cats with pancreatitis (B), and cats with pancreatic carcinoma (C). Differentially expressed proteins are noted (circles). Protein spot numbers correspond to those in Table II.

dye had reached the bottom of the plates (Protean II xi Cell; Bio-Rad Laboratories).

The gels were stained for 45 min using Coomassie blue stain (0.1% Coomassie Blue G-250, 10% glacial acetic acid, 30% methanol). Gels were then placed in destain solution (10% glacial acetic acid, 30% methanol) for 19 h in order to remove background staining.

Analysis of 2D SDS-PAGE patterns

A previous study has shown that the relationship between pixel density and protein quantity in Coomassie-stained gels is linear when assessed by specialized software (23). Therefore, the Coomassie-stained gels were scanned (HP Scanjet 5470c; Hewlett-Packard, Mississauga, Ontario) and saved as a digital data set for comparative analysis by specialized software (Image Master 2D Platinum 7.0; GE Healthcare). The relative volume of each protein spot was calculated as a percentage of the total volume of all the protein spots in each gel to help minimize potential variation due to protein loading and staining. The resulting spot volume percentage (Vol%) was used for comparison among study groups. Protein spots with consistent change among study groups were selected and the mean spot volume percentage of the protein for each group was calculated and analyzed for statistical significance using a Mann-Whitney U-test (GraphPad Prism Version 5.0; GraphPad Software, San Diego, California, USA). Values of $P < 0.05$ were considered significant.

Protein identification

Coomassie-stained protein spots of interest were excised from gels and sent to the National Research Council (NRC) in Ottawa, Ontario for identification by nanoflow high-performance liquid chromatography tandem mass spectrometry (nHPLC-MS/MS). Briefly, protein spots were destained, in-gel digested with trypsin, and the resulting digests analyzed (Q-TOF Ultima; Waters, Milford, Massachusetts, USA). The peptide mass spectra results were searched using the Mascot program against 2 databases: NCBInr protein sequence database and putative protein coding regions derived from the 2X feline genome. For the latter, peptide sequences were subsequently compared using a basic local alignment search tool (BLAST) algorithm in order to identify the protein of origin.

Results

Individual plasma proteomes contained more than 150 protein spots between pI 4 to 7 and with a molecular weight of 6.5 to 200 kD (Figure 1). Three protein spots appeared to differ among the study groups. These were identified by mass spectrometry as alpha-1-acid glycoprotein (AGP), apolipoprotein-A1 (Apo-A1), and apolipoprotein-A1 precursor (Pre Apo-A1) (Table II).

Alpha-1-acid glycoprotein (AGP) (spot 1) was not detected in 8 cats, including 4 healthy controls, 2 cats with pancreatitis, and 2 cats with pancreatic carcinoma. Cats with no detectable expression of AGP were assigned a value of zero when calculating mean values and Mann-Whitney U-test results. Differences in the expression of AGP were not found to be statistically significant, although cats with pancreatic disease tended to have higher values (Figure 2).

Apolipoprotein-A1 (Apo-A1) (spot 2) and Pre Apo-A1 (spot 3) were identified in the plasma profiles of all cats in the study. No significant differences were found in expression of Apo-A1 among the study groups, although cats with pancreatic disease, especially neoplasia, tended to have lower values (Figure 2). Conversely, Pre Apo-A1 appeared to be higher in cats with pancreatic disease, with a significant difference found between cats with pancreatic carcinoma and healthy controls. Mean values were not statistically different between cats with pancreatitis and those with pancreatic carcinoma.

Discussion

This study applied proteomic analysis, based on 2-dimensional gel electrophoresis followed by mass spectrometry. It aimed to compare the plasma protein profiles of healthy cats to those of cats with

Table II. Mean spot volume percentage of differentially expressed plasma proteins of healthy control cats and cats with pancreatitis and pancreatic carcinoma identified by 2-dimensional gel electrophoresis and mass spectrometry

Spot	Protein name	Gel MW/pI (kD/pH)	Healthy controls (Vol%)	Pancreatitis (Vol%)	Pancreatic carcinoma (Vol%)
1	Alpha-1-acid glycoprotein	59/4.0	0.080	0.539	0.335
2	Apolipoprotein-A1	30/5.5	1.524	1.306	1.066
3	Apolipoprotein-A1 precursor	20/4.8	0.604	1.559	1.940[a]

[a] Statistically significant difference in Vol% compared to healthy controls; Mann-Whitney U-test, $P < 0.05$.

MW — molecular weight; pI — isoelectric point; Vol% — spot volume percentage.

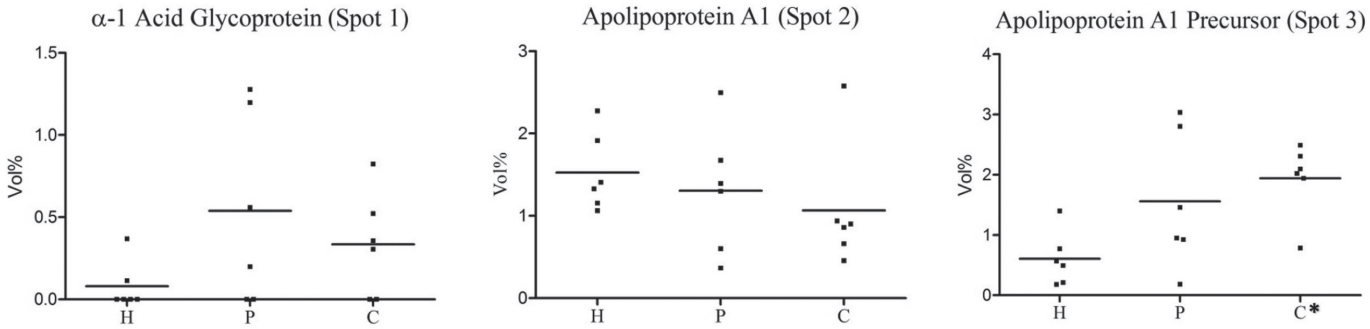

Figure 2. Spot volume percentage (Vol%) of alpha-1-acid glycoprotein, apolipoprotein-A1, and apolipoprotein-A1 precursor in healthy cats (H), cats with pancreatitis (P), and cats with pancreatic carcinoma (C). Horizontal line indicates mean Vol%.
*** Indicates a statistically significant difference in Vol% compared to healthy controls; Mann-Whitney U-test, $P < 0.05$.**

pancreatitis and pancreatic carcinoma in order to identify potential biomarkers of feline pancreatic disease. In total, 3 proteins were found to be differentially expressed: AGP, Apo-A1, and Pre Apo-A1.

Alpha-1-acid glycoprotein (AGP) is a major acute-phase protein of cats (24,25). Elevations of AGP may be seen with spontaneous and experimentally induced inflammation caused by surgery, trauma, neoplasia, and infectious disease (24,25). In this study, higher AGP concentrations tended to be found in cats with pancreatic disease, although there was considerable overlap among cats with pancreatitis, pancreatic carcinoma, and healthy controls. Furthermore, 8 cats did not express measurable amounts of AGP, including 4 healthy controls, 2 cats with pancreatitis, and 2 cats with pancreatic carcinoma. These findings suggest that other factors, such as potential variation in the degree of pancreatic inflammation and tumor burden, may influence AGP expression and hence our results. In addition, the presence of occult inflammatory disease in the 2 control cats with detectable AGP concentrations cannot be fully excluded. Levels of AGP have been reported in tumor-bearing cats, with 1 study also finding complete overlap between healthy and neoplastic cases despite a higher overall average in tumor-bearing cats (26).

In human medical research, qualitative rather than quantitative differences in AGP expression have been demonstrated, such as N-glycan changes present with both pancreatitis and pancreatic neoplasia and core fucosylation, which may be a potential marker for the diagnosis, progression, and prognosis of pancreatic carcinoma (27,28). Hypersialylation of AGP has been reported in association with feline infectious peritonitis (FIP) in cats (29), although qualitative modifications have yet to be evaluated in feline pancreatic disease. Future studies investigating potential post-translational modifications of AGP would be intriguing and may be more valuable than the quantitative determination of total AGP level in diagnosing feline pancreatic disease.

Apolipoprotein-A1 (Apo-A1) appeared lower in cats with pancreatic disease, with the spot volume values in 5/6 pancreatic carcinoma cases and 2/6 pancreatitis cases below those found in healthy control cats. Although this pattern was not found to be statistically significant, the low sample size in this study may have contributed to the lack of statistical power. Apo A-1 is a major constituent of high density lipoprotein (HDL), which is a key player in the reverse cholesterol transport pathway (16). In humans, serum Apo-A1 concentrations decrease with both pancreatic carcinoma and chronic pancreatitis, with immunohistochemical studies suggesting that Apo-A1 becomes sequestered by the pancreatic extracellular matrix with fibrosis (16,30). One study in the veterinary literature also found that Apo-A1 decreased in dogs with experimentally induced acute pancreatitis (31). Further studies on Apo-A1 concentrations, ideally by a quantitative enzyme-linked immunosorbent assay (ELISA) on serum from a larger sample of cats, would be needed to determine its relevancy as a marker of feline pancreatic disease.

Compared to Apo-A1, Pre Apo-A1 appeared higher in cats with pancreatic disease. Although there was overlap among all 3 study groups, the mean Pre Apo-A1 spot volume was significantly higher

in cats with pancreatic carcinoma than in healthy controls. One study found increased expression of Pre Apo-A1 in human pancreatic carcinoma tissue examined by 2-dimensional electrophoresis (17). Elevated concentrations of Pre Apo-A1 may also reflect increased hepatic or intestinal synthesis, possibly in response to lower circulating levels of Apo-A1 due to sequestration in the pancreas. This hypothesis is not supported by human research, however, which shows decreased hepatic Apo-A1 expression caused by inflammatory cytokines during the acute-phase response of many diseases (16). Overall, shifts in Apo-A1 and Pre Apo-A1 suggest possible alteration of lipid metabolism associated with feline pancreatic disease.

The 3 proteins (AGP, Apo-A1, and Pre Apo-A1) identified in this pilot study belong to the category of high-abundance plasma proteins. As the detection limit of our method is only 4 mg/L, biomarker proteins of low abundance may have been missed. Immunodepletion coupled with more sensitive proteomic techniques, such as fluorescent difference gel electrophoresis (2D-DIGE), would be a logical next step. Given these limitations and the low number of animals, our study should be considered preliminary. Nevertheless, we have demonstrated that serum proteomics has the potential to advance research into feline pancreatic disease. Ultimately, further studies involving more animals, as well as demonstration of repeatability and validation in an independent patient population, will be required to confirm the value of AGP, Apo-A1, Pre Apo-A1, and any newfound proteins as biomarkers for feline pancreatic disease.

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